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(54) Title: TRANSDUCTION OF GENES INTO HUMAN HEMATOPOIETIC STEM CELLS USING RECOMBINANT ADENO-ASSOCIATED VIRAL VECTORS

(57) Abstract

The invention features a method of treating a disease of hematopoietic cells in a mammal involving introducing into the mammal hematopoietic stem cells containing an adeno-associated viral vector containing a nucleic acid encoding a gene product capable of alleviating symptoms of the disease.

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TRANSDUCTION OF GENES INTO HUMAN HEMATOPOIETIC STEM CELLS USING RECOMBINANT ADENO-ASSOCIATED VIRAL VECTORS

Background of the Invention

This invention relates to gene therapy of diseases of hematopoietic cells.

Hematopoietic stem cells have two unique characteristics. First, they are pluripotent; that is, they are capable of differentiating into all mature blood cell types. Second, they are self-renewing; that is, they are capable of dividing and maintaining their pluripotentiality. Hematopoietic stem cells were first characterized by Dexter and his group (Dexter and Spencer, Ann. Rev. Cell Biol. 3:423-441, 1987). Subsequently, many other investigators have contributed to the understanding of their developmental potential, functional characteristics, and requirements for maintenance in culture (see, e.g., Dexter and Spencer, Ann. Rev. Cell Biol. 3:423-441, 1987; Lemischka et al., Cell 45:917-927, 1986). Fig. 1 illustrates the pluripotent, hematopoietic stem cell and various myeloid and lymphoid lineages that are derived from this cell (Stamatoyannopoulos et al., The Molecular Basis of Blood Diseases. (Philadelphia; W.B. Saunders Company) 88:108-109, 1994).

Summary of the Invention

We have shown that adeno-associated viral (AAV) vectors are effective at transducing genes into pluripotent hematopoietic stem cells.

Accordingly, in a first aspect, the invention features a method of treating a disease of hematopoietic cells in a mammal involving introducing into the mammal hemat poietic cells, e.g., hematopoietic stem cells, containing an AAV vector containing a nucleic acid

encoding a gene product capable of alleviating symptoms of the disease.

By "hematopoietic cells" is meant blood cells, including hematopoietic stem cells, and cells derived therefrom, including, without limitation, common lymphoid progenitor cells, T cells (e.g., helper, cytotoxic, and suppressor cells), B cells, plasma cells, natural killer cells, common myeloid progenitor cells, monocytes, macrophages, mast cells, leukocytes, basophils, neutrophils, eosinophils, megakaryocytes, platelets, and erythrocytes.

By "disease of a hematopoietic cell" is meant any condition characterized by impairment of any normal function of a hematopoietic cell. The diseases of hematopoietic cells that can be treated according to the method of the invention include genetic diseases (e.g., Adenosine Deaminase Deficiency, Fanconis' Anemia, and hemoglobinopathies, such as Sickle Cell Anemia, Thalassemias, and Hemoglobin C Disease), as well as diseases acquired by infectious or non-infectious means (e.g., Acquired Immune Deficiency Syndrome and leukemias).

By "vector" is meant a replicable nucleic acid construct. An "adeno-associated viral vector" is a vector containing AAV inverted terminal repeats (ITRs), and any modified versions thereof. AAV vectors are described in general by Kotin et al. (Kotin et al., Proc. Natl. Acad. Sci. USA 87:2211-2215, 1990). Specific examples of AAV vectors are given below.

Appropriate regulatory sequences, such as enhancers and promoters, are inserted into the vectors of the invention using standard methods known to those skilled in the art, for example, by homologous recombination (Graham et al., J. Gen. Virol. 36:59-72, 1977) or other appropriate methods (Molecular Cloning: A

Laboratory Manual, Sambrook et al., eds. Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New York, 1989). Promoters are inserted into the vectors so that they are operably linked 5' to the nucleic acid sequence encoding the gene product. By "operably linked" is meant that a gene and a regulatory sequence, such as a promoter and/or enhancer, are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s). By "promoter" is meant a minimal sequence sufficient to direct transcription. Any promoter that is capable of directing initiation of transcription in a eukaryotic hematopoietic cell may be used in the invention. For example, general promoters, such as the cytomegalovirus promoter may be used. Alternatively, hematopoietic cell type-specific promoters, e.g., globin promoters, may be used.

By "gene product" is meant a molecule that is produced as a result of transcription of a gene. Gene products include RNA molecules transcribed from a gene, as well as proteins encoded by RNA transcribed from a gene. Protein gene products that are included in the invention include, but are not limited to, anti-sickling hemoglobins, β-globin, fetal γ-globin, Fanconis' Anemia complementation gene products (e.g., the Fanconis' Anemia C gene), and adenosine deaminase. RNA gene products included in the invention include RNAs that are antisense to target RNAs. The term "antisense", as used herein, describes the interaction of an RNA gene product with a nucleic acid target in a cell in a sequence-specific manner, more particularly, the interaction of the RNA gene product with complementary target RNA sequences, resulting in inhibition of transcription or translation of the target RNA. The nucleic acid target may be a cellular nucleic acid or a nucleic acid derived from a

pathogen (e.g., a virus, such as HIV) that has infected the cell. In a preferred embodiment, the antisense RNA is antisense to HIV RNA. For a general review of the therapeutic applications of antisense nucleic acids, see, e.g., Le Doan et al., Bull. Cancer 76:849, 1989; Donick, Biochem. Pharmacol. 40:671, 1990; Crooke, Annu. Rev. Pharmacol. Toxicol. 32:329, 1992. The RNA gene product may also be a ribozyme that cleaves a target RNA which, as described above, may be a cellular nucleic acid or a nucleic acid derived from a pathogen (e.g., HIV) that has infected the cell. In a preferred embodiment, the gene product is a ribozyme that cleaves HIV RNA.

In a second aspect, the invention features a hematopoietic stem cell containing an AAV vector containing a nucleic acid encoding a gene product capable of alleviating symptoms of a disease of hematopoietic cells. The gene products contained in the hematopoietic stem cells of the invention include, but are not limited to, an anti-sickling hemoglobin, β-globin, fetal γ-globin, adenosine deaminase, the Fanconis' Anemia C gene product, RNA that is antisense to HIV RNA, or a ribozyme that cleaves HIV RNA. Mammals that can be treated using the methods of the invention include, but are not limited to, humans, cows, dogs, cats, sheep, goats, rabbits, rats, guinea pigs, hamsters, and mice.

In a third aspect, the invention features an adeno-associated viral vector made in a semi-packaging cell line, and containing a nucleic acid encoding a therapeutic gene product, for use in therapy.

In a fourth aspect, the invention features a hematopoietic cell (e.g., a hematopoietic stem cell) containing an adeno-associated viral vector made in a semi-packaging cell line, and containing a nucleic acid encoding a therapeutic gene product, for use in therapy.

In a fifth aspect, the invention features the use of an adeno-associated viral vector for the preparation of a medicament for use in therapy for a disease of hematopoietic cells in a mammal. The viral vector is made in a semi-packaging cell line and contains a nucleic acid encoding a gene product capable of alleviating a symptom of the disease.

In a sixth aspect, the invention features a hematopoietic cell containing an adeno-associated viral vector for use in therapy for a disease of hematopoietic cells in a mammal. The adeno-associated viral vector is made in a semi-packaging cell line and contains a nucleic acid encoding a gene product capable of alleviating a symptom of the disease.

The adeno-associated viral vectors used in the invention may be produced from the semi-packaging cell line at a titer of at least 10^7 or 10^8 plaque-forming units/milliliter. The semi-packaging cell line may contain the rep or cap genes of adeno-associated virus on an episomal vector.

The advantages of using AAV vectors in gene therapy methods include (1) AAV are completely non-pathogenic, (2) AAV have a broad host range, (3) AAV particles can be concentrated to titers of 10^9, (4) AAV inverted terminal repeats (ITRs) do not contain enhancers that can activate host genes (e.g., oncogenes) upon viral insertion, and (5) AAV are capable of infecting quiescent cells.

Other features and advantages of the invention will become apparent from the following detailed description, the drawings, and the claims.

Detailed Description

The drawings are first described.

Drawings

Fig. 1 is an illustration of the pathways of differentiation of hematopoietic stem cells.

Figs. 2A and 2B are schematic representations of pEBAVrc and pEBAVcmv/lac vectors, respectively.

Fig. 3 is agarose gel analysis of PCR reactions containing DNA extracted from twenty two separate BFU-E (burst-forming units-erythroid) which differentiated from hematopoietic stem cells transfected with pEBAVcmv/lac that were taken from a homozygous sickle cell patient .

Fig. 4 is an illustration of 5′ HS 1-5 α1 and HS 1-5 β^{AS} constructs. One hundred kilobases of the human β-globin locus and 35 kilobases of the human α-globin locus are illustrated. Cosmids containing HS 1-5 α1 and HS 1-5 β^{AS} were constructed by fusing either the α1 gene or a recombinant anti-sickling β-globin gene downstream of the β-globin locus control region (LCR). The 26 kb inserts were purified from vector sequences, mixed at a 1:1 molar ratio (final DNA concentration was 2 ng/μl), and co-injected into fertilized mouse eggs. Transgenic lines displaying high-level, balanced expression of the transgenes were established.

Figs. 5A-5D are graphs of chromatographs of hemolysates and HPLC-purified anti-sickling hemoglobins. Fig. 5A is a graph of a chromatograph of hemolysate obtained from transgenic mice expressing Hb AS1. Hemoglobins were separated by non-denaturing HPLC. Twenty eight percent of the hemoglobin in erythrocytes of these animals is recombinant human $\alpha\beta^{AS1}$. Fig. 5B is a graph of denaturing HPLC analysis of $\alpha\beta^{AS1}$ purified from the hemolysate shown in Fig. 5A. Purification was performed by preparative isoelectric focusing (IEF). Approximately 10% of the β-globin chains were of murine origin. Fig. 5C is a graph of a chromatograph of hemolysate btained from transgenic mice expressing Hb AS2. Hemoglobins were separated by non-denaturing HPLC.

Eighteen percent of the hemoglobin in the erythrocytes of these animals is recombinant human $\alpha\beta^{AS2}$. **Fig. 5D** is a graph of denaturing HPLC analysis of $\alpha\beta^{AS2}$ purified from the hemolysate shown in Fig. 5C. Purification was performed by preparative IEF. This hemoglobin lacks any contaminating murine globins. Hemoglobins purified by preparative IEF were used in all subsequent experiments.

Figs. 6A-6D are oxygen equilibrium curves (OECs) for purified human anti-sickling hemoglobins. **Fig. 6A** is an OEC curve for Hb AS1 at pH 7.0 in 0.1 M potassium phosphate (KPO_4) buffer at 20°C. **Fig. 6B** is an OEC curve for Hb AS1 under the same conditions as those described in Fig. 6A, with the addition of 2 mM 2,3-diphosphoglycerate (DPG). **Fig. 6C** is an OEC curve for Hb AS2 at pH 7.0 in 0.1 M KPO_4 buffer at 20°C. **Fig. 6D** is an OEC curve for Hb AS2 under the same conditions as those described in Fig. 6C with the addition of 2 mM 2,3-DPG.

Figs. 7A-7B are graphs showing polymerization delay times for deoxygenated mixtures of human hemoglobins. **Fig. 7A** shows delay times for hemoglobin mixtures containing 100% Hb S or 75% Hb S, together with 25% Hb A, Hb AS1, Hb AS2, or Hb F. Curves were determined at a hemoglobin concentration of 60 mg/dl using the temperature jump method (Adachi et al., J. Biol. Chem. 254:7765, 1979). The delay time is an indication of the ability of a hemoglobin to disrupt the polymerization of Hb S. The delay time of Hb AS1 is between that of Hb A and Hb F, while the delay time of Hb AS2 is similar to that of Hb F at this hemoglobin concentration. **Fig. 7B** shows delay time vs. hemoglobin concentration. The progression of the plots from left to right demonstrates the increased Hb concentrations which are required for polymerization to occur in the presence

of the various non-S hemoglobins. The delay time plots for Hb AS2 and Hb F overlap, indicating that the anti-polymerization activities of Hb AS2 and Hb F are virtually identical.

Fig. 8 is an illustration of a retroviral vector useful for the production of anti-sickling hemoglobin.

Gene Transfer into Hema letic Stem Cells

We have shown th. vectors are capable of transducing genes into hematopoietic stem cells. Hematopoietic stem cells differentiate into a large number of different cells (Fig. 1), including common lymphoid progenitor cells, T cells (e.g., T-helper cells, cytotoxic T cells, and suppressor T cells), B cells, plasma cells, natural killer cells, common myeloid progenitor cells, monocytes, macrophages, mast cells, leukocytes, basophils, neutrophils, eosinophils, megakaryocytes, platelets, and erythrocytes. Gene transfer into hematopoietic stem cells, therefore, potentially results in gene transfer into all of these cells. Thus, any disease that can be treated by the expression of a transgene in any of these cells can be treated using the methods of the present invention. For example, hemoglobinopathies, such as β-thalassemia and sickle cell anemia, can be treated by using, e.g, the β-globin gene (Karlsson et al., Ann. Rev. Biochem. 54:1071-1108, 1985, and references therein), while α-thalassemia can be treated by using the α-globin gene. Sickle cell anemia can also be treated by using an anti-sickling hemoglobin gene (see below) or a γ-globin gene. Fanconis' anemia can be treated using the Fanconis' C gene, or genes from other Fanconis complementation groups (dos Santos et al., Stem Cells 12:142-153, 1994). Further, adenosine deaminase deficiency (ADA) can be treated by using the adenosine deaminase gene (Blaese et

al., Hum. Gene Ther. 4:521-7, 1993); and acquired immun deficiency syndrome (AIDS) can be treated by using anti-sense or ribozyme sequences designed to inhibit HIV replication (Tsuchuhashi et al., Science 262:99-102, 1993; Lisziewicz et al., Proc. Natl. Acad. Sci. USA 90:8000-4, 1993) in the methods of the invention.

AAV vectors are vectors that contain AAV inverted terminal repeats (ITRs). AAV belongs to the genus Dependovirus (family parvoviridae), and is also known as adenosatellite and dependovirus. These viruses are antigenically unrelated to adenovirus, but require the presence of adenovirus in order to replicate. There are at least four serotypes, all sharing common antigens. Dependoviruses are characterized in that they (1) are small and containing single-stranded DNA, (2) have virions of 18 to 26 nm in diameter, (3) are not enveloped, (4) are ether-resistant, (5) have capsids with cubic symmetry (with 32 capsomeres), and (6) their replication and assembly occur in the nucleus of infected cells.

Appropriate promoters and genes are introduced into the vectors of the present invention using standard methods in the art (see, e.g., Molecular Cloning: A Laboratory Manual, Sambrook et al., eds. Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New York, 1989). Any promoter that is sufficient to direct the initiation of transcription in a hematopoietic cell may be used in the invention. For example, a preferred non-tissue specific promoter is the cytomegalovirus (CMV) promoter (DeBernardi et al., Proc. Natl. Acad. Sci. USA 88:9257-9261, 1991, and references therein). Any hematopoietic cell-type specific promoter may also be used in the invention. For example, globin promoters, such as the β-globin promoter (Karlsson et al., Ann. Rev.

Biochem. 54:1071-1108, 1985, and references therein) may be used.

Any appropriate source may be used to obtain the hematopoietic stem cells used in the invention. Preferably, the hematopoietic stem cells are obtained from the patient into which the stem cells are to be transplanted after AAV-infection. Hematopoietic stem cells are obtained from patients and, after infection by AAV vectors, are introduced into patients using standard methods in the art (see, e.g., United States Patent 5,061,620; Rubenstein et al., (eds.) Scientific American Medicine, Scientific American, Inc. (New York, 1978), and references therein).

The most stringent experimental criterion for hematopoietic stem cells is the ability of these cells to reconstitute the entire blood system of a lethally irradiated animal. This experiment obviously cannot be carried out in humans and therefore other assays have been developed to demonstrate the pluripotentiality and self-renewing characteristics of human hematopoietic stem cells. The most widely accepted assay for human hematopoietic stem cells is the Long Term Culture-Initiating Cell (LTC-IC) assay, which involves culturing bone marrow cells for 5 weeks and then analyzing the developmental potential of surviving cells by plating these cells in semi-solid medium (Eaves et al., J. Tiss. Cult. Meth. 13:55-62, 1991). After 14 days in semi-solid medium, colonies of all cell lineages are detectable if pluripotent, self-renewing cells were present when the long term culture was initiated.

The LTC-IC assay has been used to demonstrate that retroviral vectors are capable of transducing genes into human hematopoietic stem cells (Hughes et al., J. Clin. Invest. 89:1817-1824, 1992). AAV vectors, which, as described above, have several advantages for use in gene

therapy, have not been previously tested in this system. The receptor for AAV is not known; therefore, it was impossible to predict whether the virus would infect hematopoietic stem cells. We purified human hematopoietic stem cells by the procedures described below and infected these cells with recombinant AAV.

Production of high titer, recombinant AAV

We developed a packaging line that is capable of producing a high titer (10^7 pfu/ml) stock of AAV-derived vector containing a CMV/*lacZ* reporter gene. The "semi-packaging" cell line was established with a silent episomal vector (pEBAVrc; Fig. 2A). This vector contains an EBV replication origin and an EBNA1 gene. This arrangement enables the vector to be established as an episome and replicate to high copy numbers. The rep-cap genes of AAV are inserted into the vector with their natural control elements; therefore, these genes are activated only in the presence of adenovirus early gene products. This arrangement provides a switch-system to regulate the expression of the rep gene, which has been found to have a cytostatic effect. The vector also contains a hygromycin resistance gene to provide selection for cells that contain the vector. The plasmid sequences of the vector contain an ampicillin resistance gene and a bacterial replication origin for replicating the DNA in bacteria.

The AAV vector containing the lacZ gene was inserted into a similar vector, pEBAVcmv/lac (Fig. 2B). This vector contains a CMV immediate-early transcriptional cassette containing the lacZ gene which is flanked by the two ITR sequences of AAV.

To establish the semi-packaging cell line, HeLa cells in 100 mm culture dishes were transfected with pEBAVrc. HeLa cells were seeded 24 hours prior to transfection, and all wed to reach 50-70% confluence at

the time of transfection. Cells were washed once with serum-free DMEM (Gibco). After removal of the medium, 8 ml of DMEM containing 10% fetal calf serum was added to the culture on the next day. At 12 hours after this medium change, cells were trypsinized and passed to three 100 mm dishes in DMEM containing 10% fetal calf serum and 200 u/ml of Hygromcyin (Sigma). Special care should be taken to allow cells to settle at the bottom of the plate individually. Cells were selected with hygromycin for 10 to 15 days when individual colonies began to grow. Colonies from 3 to 4 dishes were trypsinized and pooled into one 100 mm dish. Cells were kept at 60-70% confluence in medium containing 200 u/ml of hygromycin. When 90% confluence was reached cells were transferred into four 100 mm dishes in DMEM containing 10% DMEM without hygromycin. At 24 hours post-seeding, each 100 mm dish of cells was transfected with 5 μg each of pEBAVrc and pEBAVcmv/lac. At 3 hours after the addition of the transfection medium, wild-type adenovirus (HuAd5) was added to the cells at an MOI of 10, without removal of the transfection medium. At 12 hours after the transfection, the medium was changed to DMEM containing 5% fetal calf serum and incubated for three days or until most of the cells showed cytopathic effects. All of the media except 1 ml was removed from each 100 mm dish. Cells were scraped off the plate and transferred into a 10 ml screw-cap tube. This viral lysate was stored at -100°C until further treatment.

The viral lysate was thawed on ice, and sonicated for 10-15 minutes with pulse ultrasound. The tube was put back on ice periodically to prevent the sample from heating up. The viral lysate was then spun at 15,000 rpm for 10 minutes in a JL-20 centrifuge (Beckman). The supernatant was removed and aliquoted into freezing wells of 10 ml each and stored at -100°C. For infecting of

hematopoietic stem cells, it was not necessary to remove or inactivate the adenovirus since stem cells lack receptor for adenovirus. For infection of cells that are susceptible to adenovirus infection, adenovirus should be removed by chromatography, of CsCl gradient centrifugation.

The titer of the virus was determined by the rate of transduction of HeLa cells with the lacZ gene. One, two, and three ml of the viral stock were used to infect HeLa cells in 24-well plates. At 24 hours post infection, the cells were fixed with 0.1% glucoraldehyde at 4°C for 2 min, and washed thoroughly with DMEM. Cells were then stained with 0.3% X-gal with standard procedures. Percentages of the cells that stained blue were determined by light microscopy. There were 2 x 10^5 HeLa cells in each well and one half of the cells (1 x 10^5) stained blue. Therefore, the viral titer was at least 10^8/ml.

The transfection procedure was as follows. For each 100 mm dish, 8-10 μg DNA was dissolved in 2 ml of DMEM or transfection medium from BRL. 90-100 μl of lipofectamine (BRL) was suspended in 2 ml of DMEM. The lipid containing medium was mixed with the DNA containing medium slowly and dropwise, while the tube was gently rotated. After mixing well by gently rotating the tube, the transfection mixture was incubated for 30 minutes at room temperature. The mixture was then diluted with DMEM to a total volume of 8 ml. The mixture was then added to the cells, which had been washed once or twice with DMEM without serum. Care should be taken to not allow the cells to dry between each wash.

Purification of human hematopoietic stem cells

Bone marrow was obtained from individuals following the IRB approved protocol. Mononuclear cells were purified using density gradient centrifugation using

standard procedures. The mononuclear bone marrow cells were isolated by Ficoll/Hypaque density gradient centrifugation (average yield 1-2 x 10^8 cells). The recent development of magnetic activated cell sorting (MACS) allowed for rapid enrichment of CD34+ cells (Kato and Radbruch, Cytometry 14:384-392, 1993). The cells were labeled with anti-CD34+ antibodies that were conjugated with magnetic particles and run through the magnetic separation column from Miltenyi Biotec (Sunnyvale, CA). A single passage of mononuclear human bone marrow cells through the magnetic column yielded CD34+ cells that were greater than 90% pure. Hematopoietic stem cells were further enriched by selecting for and against various surface molecules according to principles outlined by Baum et al. (Proc. Natl. Acad. Sci. USA 89:2804-2808, 1992). The progenitors were labeled with FITC conjugated antibody that recognizes a different CD34+ epitope than the magnetic bead conjugated anti-CD34 antibody. Lin- cells are isolated by a negative selection using a cocktail of PE labeled antibodies that recognize myeloid, B cells, T cells, and NK cell markers (CD 2, CD 14, CD 19, CD 56). Thy+ cells were isolated by positive selection with biotinylated, anti-human Thy mouse monoclonal antibody that was labeled with steptavidin-conjugated, Texas red dye.

Infection of hematopoietic stem cells

Ten thousand CD34+, Lin-, Thy+ cells were suspended in 200 μl of DMEM without serum and dispensed into a single well of a 96 well plate. Ten microliters of the recombinant virus (AAV cmv/lacZ; 10^8 u/ml) were added to the cells and incubated at 37°C for 16 hours. The cells were then washed 3 times with DMEM without serum and plated into long term culture as described by Eaves et al. (J. Tiss. Cult. Meth. 13:55-62, 1991).

Analysis f cells

After 6 weeks in long term culture, cells were removed and plated in methylcellulose for clonogenic progenitor assays (Eaves and Eaves, Blood 52:1196-1210, 1978). BFU-E were picked after 14 days, DNA was extracted and PCR was performed with primers specific for lacZ. PCR products were run on agarose gels, blotted to nitrocellulose and hybridized with radiolabeled, lacZ sequences. Fig. 3 demonstrates that at least 5 of 22 (22%) BFU-E contained the transduced lacZ gene.

Anti-Sickling β-globin Genes Designed to Inhibit Hb S Polymerization

The molecular basis for sickle cell disease is an A to T transversion in the 6th codon of the human β-globin gene. This simple transversion changes a polar glutamic acid residue to a non-polar valine (Ingram et al., Nature 178:792, 1956; Ingram et al., Nature 180:326, 1957) in the β-globin polypeptide and, thus, drastically decreases the solubility of this hemoglobin (termed Hb S). When the intracellular concentration of Hb S is high and the partial pressure of oxygen in the capillary beds is low, the non-polar valine, which is on the surface of the hemoglobin molecule, interacts with two other non-polar residues on the surface of a second hemoglobin molecule, and initiates aggregation (Padlan et al., J. Biol. Chem. 260:8280-8291, 1985; Wishner et al., J. Mol. Biol. 98:179-194, 1975). Once approximately 10 hemoglobin monomers interact, long polymers rapidly accumulate, and complex 14-stranded fibers are formed (Crepeau et al., Nature 274:616-617, 1978; Dykes et al., J. Mol. Biol. 130:451-472, 1979; Eaton et al., Blood 70:1245-1266, 1987; Hofrichter et al., Proc. Natl. Acad. Sci. USA 71:4864-4868, 1974). The formation of these fibers reduces the flexibility f red blood cells, leading to the cclusion of small capillari s. Intracellular fiber

formation also results in erythrocyte membrane damage and increased red cell lysis (Noguchi et al., Blood 58:1057, 1981; Brittenham et al., Blood 65:183, 1985). The ensuing disease is characterized by a chronic hemolytic anemia, episodes of severe pain, and tissue damage that can result in stroke, kidney failure, heart disease, infection, and other complications (Bunn et al., Hemoglobin: Molecular, Genetic, and Clinical Aspects. (W. B. Saunders, Philadelphia, 1986)).

Recombinant hemoglobins which contain anti-sickling mutations can be used to inhibit Hb S polymerization, and thus facilitate gene therapy of sickle cell anemia. The glutamic acid to valine change at the 6th position of the β^s polypeptide creates a non-polar surface that readily interacts with a natural hydrophobic pocket in the β chain of a second tetramer. This natural pocket is formed primarily by phenylalanine (phe) at position 85 and leucine (leu) at position 88. This interaction leads to the formation of the complex 14-stranded fibers described above (Bunn et al., Hemoglobin: Molecular, Genetic, and Clinical Aspects, 1986, W.B. Saunders, Philadelphia). The structure of the fiber that forms in sickle erythrocytes was derived from X-ray diffraction studies of Hb S crystals (Edelstein, J. Mol. Biol. 150:557, 1981). Hb S tetramers are composed of two α-globin subunits (α_2) and two β^s-globin subunits ($\beta^s{}_2$), and form the characteristic double-stranded fibers. Interactions along the long axis of the fiber are termed axial contacts, while interactions along the sides of tetramers are lateral contacts (Bunn et al., Hemoglobin: Molecular, Genetic, and Clinical Aspects. (W.B. Saunders, Philadelphia, 1986)). The β6 valine plays a critical role in the lateral contact by interacting with the hydroph bic residues β85 phenylalanine and β88 leucine. Acc rdingly, to interfere with detrimental Hb S

polymerization, this interaction and, thus, hydrophobic pocket formation should be disrupted. Because Hb A ($\alpha_2\beta_2$) has these same hydrophobic residues and is readily incorporated into sickle fibers, it cannot be used for this purpose. Moreover, although disruption of this pocket represents the best approach for inhibiting Hb S polymerization, certain strategies have detrimental side effects. For example, although amino acid substitutions at β85 phe and β88 leu would interfere with pocket formation, these amino acids are also important for correct positioning of the heme moiety, and cannot be mutated without severely altering oxygen affinity (Dickerson et al., Hemoglobin: Structure, Function, Evolution, and Pathology. (Benjamin/Cummings, Menlo Park, CA, 1983)).

A better approach for inhibiting Hb S polymerization involves making a β87 threonine (thr) to glutamine (gln) substitution that disrupts the hydrophobic pocket, without inhibiting β-globin function (Perutz et al., Nature 219:902-909, 1968). The long side chain of glutamine prevents the β6 Val from interacting with the hydrophobic pocket. Human γ- and δ-globin polypeptides both have such a glutamine at position 87, and both Hb F ($\alpha_2\gamma_2$) and Hb A2 ($\alpha_2\delta_2$) have potent anti-sickling activity (Nagel et al., Proc. Natl. Acad. Sci., USA 76(2):670-672, 1979). Another naturally occurring human hemoglobin, designated Hb D Ibadan, also has anti-sickling activity (Watson-Williams et al., Nature 205:1273, 1965). This hemoglobin has a lysine at position 87 and its long side chain also projects across the hydrophobic pocket and inhibits interactions with the β6 Val.

Preferably, to produce a recombinant anti-sickling hemoglobins, the mutations described above (which interfere with a major lateral contact) are combined with

a second mutation which interferes with an axial contact. The side chains of the amino acids lysine-17 (lys), asparagine-19 (asn), and glutamic acid-22 (glu), project to form a surface which stabilizes the axial contact with another sickle hemoglobin tetramer (Dickerson et al., Hemoglobin: Structure, Function, Evolution, and Pathology. (Benjamin/Cummings, Menlo Park, CA, 1983)). Although mutations at residues 17 or 19 are detrimental, amino acid 22 can be mutated from glutamic acid to alanine (ala) without an alteration in hemoglobin function (Bowman et al., Biochemical and Biophysical Research Communications 26(4):466-470, 1967; Bunn et al., Hemoglobin: Molecular, Genetic, and Clinical Aspects. (W.B. Saunders, Philadelphia, 1986)). The negative charge of the glutamic acid side chain at this position plays a key role in stabilizing the axial contact because it interacts with the positively charged imidazole group of a histidine at position 20 in the α chain of the neighboring tetramer. The shorter nonpolar alanine side chain fails to stabilize this interaction, thus disrupting the axial contacts between sickle hemoglobin tetramers. Hb AS2 contains a glutamine at position 87 together with an alanine at position 22. Hb AS1 has the same β22 alanine and asparagine at β80 is replaced by lysine. This β80 lysine significantly inhibits sickling when present as a single site mutation in Hb A (Nagel et al., Nature 283:832, 1980). The following 27-mer oligos were used for mutagenesis at the indicated amino acids in β-globin: β22, GTGAACGTGGATGCCGTTGGTGGTGAG (SEQ ID NO: 1); β80, GCTCACCTGGACAAGCTCAAGGGCACC (SEQ ID NO: 2); β87, GGCACCTTTGCCCAGCTGAGTGAGCTG (SEQ ID NO: 3).

Another anti-sickling mutation in the human β-globin gene useful in the invention is the Hb G Szuhu mutation, a β80 asn to lys mutation which has significant anti-sickling activity (Nagel et al., Proc. Natl. Acad.

Sci. USA 76(2):670-672, 1979), but which does not impair hemoglobin function (Kaufman et al., Human Heredity 25:60-68, 1975). This mutation is preferably combined with the β22 glu to ala mutation described above.

Alternatively, an α-globin mutation may be utilized to inhibit Hb S polymerization. One example of such an α-globin mutation is provided by the hemoglobin designated Hb Montgomery (Brimhall et al., Biochim. Biophys. Acta. 379(1):28-32, 1975), which contains an α48 leucine to arginine mutation. The 54 year old patient from which this mutation was isolated was homozygous for β^s, but had no history of painful sickle cell crises, jaundice, leg ulcers, or stroke, and was only mildly anemic (Prchal et al., Am. J. Med. 86(2):232-236, 1989).

Anti-sickling hemoglobin AS3 combines the mutations at β22 and β87, which are present in anti-sickling hemoglobin AS2, with an additional mutation which lowers the oxygen affinity of the recombinant hemoglobin. The goal is to produce an anti-sickling hemoglobin which delivers oxygen to tissues prior to sickle hemoglobin (Hb S). We have termed this concept "preferential deoxygenation." If the anti-sickling hemoglobin delivers oxygen preferentially, Hb S will remain oxygenated and, therefore, will not polymerize.

The mutation which was selected to lower the oxygen affinity of the anti-sickling hemoglobin is a change from asparagine to lysine at position 108 of the β-globin chain. This is the mutation which is present in the naturally-occurring Hb Presbyterian (Moo-Penn et al., FEBS Letters 92:53-56, 1978). Hb AS3 has the following three mutations: (1) β22 glutamic acid to alanine, (2) β87 threonine to glutamine, and (3) β108 asparagine to lysine.

Two additional anti-sickling hemoglobins, AS4 and AS5, have been made which combine the mutations present

in Hb AS2 at β22 and β87, with additional mutations which cause the β-globin subunit to become more negatively charged. In red blood cells, surface charge is a key determinant of the ability of α-globin and β-globin monomers to associate with each other to form dimers (Bunn, Blood 69:1-6, 1987). The alpha subunit is somewhat positively-charged, while the beta subunit is somewhat negatively charged. By increasing the negative charge on the β-globin subunit, it is possible to increase affinity for the α-globin subunit. Introduction of an additional negative charge in the anti-sickling hemoglobin will provide β^{AS} polypeptides with a competitive advantage for interacting with α-globin polypeptides. Consequently, $\alpha_2\beta^{AS}{}_2$ tetramers will form more efficiently than $\alpha_2\beta^{S}{}_2$ tetramers.

Anti-sickling hemoglobins Hbs AS4 and AS5 combine the mutations present in AS2 with a mutation which increases the negative charge on the β-globin subunit. One mutation which increases the negative charge on the β-globin subunit, but which does not affect the normal functioning of the hemoglobin molecule, is a change from lysine to glutamic acid at position 95. This mutation occurs naturally and is known as Hb N-Baltimore. The resulting change in charge is -2, since a positively-charged lysine is replaced by a negatively-charged glutamic acid. This change in charge also allows Hb AS4 and Hb S to be distinguished by isoelectric focusing. Hb AS4 has the following three mutations: (1) β22 glutamic acid to alanine, (2) β87 threonine to glutamine, and (3) β95 lysine to glutamic acid.

An additional mutation which occurs naturally and which is known to increase the ability of the β-globin subunit to compete for the α-globin subunit is known as Hb J-Baltimore. This mutation consists of a change from glycine to aspartic acid at position 16 of the β-globin

subunit. While this mutation adds only one additional negative charge to the β-globin chain (compared to the two negative charges added by the N-Baltimore mutation described above), the location of the negative charge is significant. In fact, Hb J-Baltimore competes even more effectively than Hb N-Baltimore for the α-globin subunit. Hb AS5 has the following three mutations: (1) β16 glycine to aspartic acid, (2) β22 glutamic acid to alanine, and (3) β87 threonine to glutamine.

The invention includes the use of anti-sickling hemoglobins that contain any combinations of the individual mutations described above. For example, the β108, β95, and β16 mutations may occur either alone, in combination with the β22 mutation, or in combination with the β22 mutation and either the β80 or either of the above-described β87 mutations.

Mutagenesis of Human α- and β-globin Genes

Mutations may be introduced into the normal human α- and β-globin genes by site-directed mutagenesis. For example, a 3.8 kb BglII-EcoRI fragment containing the human α-globin gene or a 4.1 kb HpaI-XbaI fragment containing the human β-globin gene may be cloned into the pSELECT plasmid (Lewis et al., Nucl. Acids. Res. 18:3439-3443, 1990; pSELECT is available from the American Type Culture Collection, Rockville, Maryland, ATCC# 68196) using standard methods (see e.g., Maniatis et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Oligonucleotide mutagenesis is performed, e.g., as described by Lewis et al. (Nucl. Acids. Res. 18:3439-3443, 1990). In this procedure, an oligonucleotide which corrects a mutation in the ampicillin resistance gene in the pSELECT plasmid is used simultaneously with one or more ligonucleotides designed to create mutations in the gl bin gene insert.

Briefly, *E. coli* (JM109; ATCC 53323) containing the pSELECT plasmid with globin gene inserts are infected with helper phage (M13K07). After growing the culture overnight (about 12-16 hours), phage obtained from the supernatant are extracted with phenol:chloroform, and single-stranded DNA is isolated by standard methods. Oligonucleotides containing each of the mutations are annealed to single-stranded DNA together with the wild-type ampicillin oligonucleotide, and these primers are extended with Klenow for about 90 minutes at 37°C. Double-stranded DNA is transformed into *E. coli* (BMH 71-18 mutS), and the culture is grown overnight in L- broth containing 75 μg/ml ampicillin. DNA obtained from rapid lysis preparations of these cultures is transfected into *E. coli* (JM109), and colonies are selected on ampicillin plates (75 μg/ml). Double-stranded DNA obtained from rapid lysis preparations of these colonies is sequenced (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977) using oligonucleotide primers located upstream of the mutagenic oligonucleotides. Mutants are clearly identified by comparison to wild-type sequence.

Construction of Cosmid Clones

The DNA constructs used for microinjection to produce transgenic animals are shown in Fig. 4, and were prepared as described by Behringer et al. (Science 245:971, 1989), except that the gene for sickle hemoglobin is replaced with genes encoding anti-sickling hemoglobins. Mutations are introduced into the human β-globin gene by site-specific mutagenesis, as described above, and the mutant sequences are inserted downstream of a 22 kb DNA fragment containing the DNAse hypersensitive sites 1-5 (5′ HS 1-5) of the β-globin LCR

(Lewis et al., Nucleic Acids Res. 18:3439, 1990), as described in further detail below.

In order to construct cosmid clones containing mutant α- and β-globin genes, the mutant genes are excised from pSELECT plasmids and subcloned into "right arm" plasmids containing a Cos site. Specifically, a 1.2 kb NcoI-XbaI fragment from the α-globin pSELECT plasmids and a 1.4 kb ClaI-BamHI fragment from the β-globin pSELECT plasmids are inserted into right arm plasmids in place of the corresponding α- and β-globin gene wild-type fragments. The α-globin right arm plasmids are digested with ClaI and MluI, and 4.8 kb fragments containing mutant α-globin genes which are linked to Cos sites are purified by agarose gel electrophoresis. The β-globin right arm plasmids are digested with ClaI and HindIII, and 6.5 kb fragments containing mutant β-globin genes which are linked to Cos sites are purified similarly. Cosmids containing these fragments are constructed in four way ligations (Ryan et al., Genes Dev. 3:314-323, 1989). The left arms are 9.0 kb MluI-SalI fragments obtained from the cosmid vector pCV001 (Lau et al., Proc. Natl. Acad. Sci. U.S.A. 80:5225-5229, 1983). This fragment contains a Cos site, an ampicillin resistance gene, a ColE1 origin and the SVneo gene. The two internal fragments are a 10.7 kb SalI-KpnI fragment containing DNase I super-hypersensitive (HS) sites V, IV and III, and a 10.9 kb KpnI-ClaI fragment containing HS II and I. The four fragments are ligated together in a 2:1:1:2 molar ratio of vector arms to inserts and packaged (Packagene; Promega, Madison, WI). *E. coli* ED8767 is infected with the packaged cosmids and is plated onto ampicillin plates. Large scale cultures of ampicillin resistant colonies are grown, and cosmids are prepared by standard procedures.

Transgenic animal assays - Characterization of anti-sickling hemoglobins

The effects of anti-sickling hemoglobin can be analyzed using transgenic animals. Cosmid DNA is prepared by standard procedures. HS I-V α and HS I-V β cosmids containing the mutations described above are either injected directly into fertilized mouse eggs, or the constructs are digested with SalI and insert DNA is separated from plasmid DNA by agarose gel electrophoresis prior to injection. The injected eggs and transferred to pseudopregnant foster mothers (Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985), and transgenic progeny are identified by Southern blot hybridization of tail DNA. Similarly, large animal eggs can be injected with the same constructs and transferred to foster mothers as described by Pursel et al. (Science 244:1281-1288, 1989). Typically, human α- and β-globin genes are cloned into expression vectors designed to direct high levels of α- and β-globin synthesis in erythroid cells of transgenic animals. These constructs are co-injected into fertilized mouse eggs and expression is analyzed in transgenic animals that develop.

Blood collected from transgenic animals is washed with saline, and hemolysates prepared as described by Ryan et al. (Science 245:971-973, 1990). Hemoglobin is analyzed on isoelectric focusing (IEF) gels (Ryan et al., Science 245:971-973, 1990) to demonstrate that a complete human hemoglobin is formed in adult erythrocytes, and to identify transgenic animals which synthesize high levels of human hemoglobin (Ryan et al., Science 247:566, 1990; Behringer et al., Science 245:971, 1989). Human hemoglobin bands are excised from IEF gels and analyzed on urea cellulose acetate strips to demonstrate that the human hemoglobin band is composed of human α- and β-globin polypeptides. It is noted that if human

hemoglobin is difficult to separate from endogenous hemoglobins, mutations that increase or decrease the isoelectric point (pI) of human hemoglobin can be introduced into the α- and β-globin genes. Increases in pI are accomplished by introducing basic (positively charged) amino acids into the protein, while decreases in pI are accomplished by introducing acidic (negatively charged) amino acids. These charged amino acids are introduced at positions which do not disturb the structure or function of the protein. Oxygen equilibrium curves (OECs) of human hemoglobin purified from the transgenic mice are determined as described by Ryan et al. (Science 247:566-568, 1990).

The anti-sickling properties of the AS hemoglobins (purified from erythrocytes of the above-described transgenic animals) can be quantitated by *in vitro* solubility assays as described, e.g., by Benesch et al. (J. Biol. Chem. 254:8169, 1979). Briefly, the anti-sickling hemoglobin is mixed with Hb S. The solution is cooled to 0°C, deoxygenated, and then incubated at 30°C for 2 to 3 hours. Insoluble polymers are pelleted by ultracentrifugation, and the concentration of hemoglobin in the supernatant is determined spectrophotometrically. The solubility of mutant hemoglobin/Hb S mixtures is compared with Hb A/Hb S and Hb F/Hb S solutions.

Characterization of anti-sickling hemoglobins AS1 and AS2 produced in transgenic mice

Transgenic lines expressing AS1 or AS2 were established, and hemolysates obtained from several animals were analyzed by anion exchange high performance liquid chromatography (HPLC) to quantitate the amounts of human, mouse, and hybrid hemoglobins (Ip et al., Anal. Biochem. 156:348, 1986; Hemoglobin tetramers were separated by anion exchange HPLC utilizing a Synchropak AN 300 (4.6 mm x 25 mm) c lumn (SynChrom, Lafayette,

IN)). Figs. 5A and 5C show that 28% of total hemoglobin was Hb AS1 in one $\alpha\beta^{AS1}$ transgenic line, and 18% of total hemoglobin was Hb AS2 in one $\alpha\beta^{AS2}$ transgenic line. Hemoglobins AS1 and AS2 were isolated by preparative IEF (Behringer et al., Science 245:971, 1989) and the purity of the human hemoglobins was assessed by denaturing reverse phase (HPLC) which separates the α- and β-globin subunits (Adachi et al., J. Chromat. 419:303, 1987. Mouse and human globins were separated by RP-HPLC using a Dionex Series 4500i HPLC system (Sunnyvale, CA). Approximately 25-30 μg of hemoglobin was injected into a Vydac C4 reversed phase column (4.6 mm x 250 mm; Hibernia, CA) and eluted with a linear gradient of acetonitrile and 0.3% trifluoroacetic acid as described in Shelton et al., J. Liq. Chrom. 7:1969, 1977). Figs. 5B and 5D show that Hb AS1 was approximately 90% pure, while Hb AS2 was purified to homogeneity.

Oxygen equilibrium curves (OEC) for purified Hb AS1 and Hb AS2 are illustrated in Figs. 6A and 6C (Asakura et al. in Oxygen Transport in Red Blood Cells, C. Nicolau Ed. (Pergamin, New York, 1986). Oxygen equilibrium curves were measured with a Hemox Analyzer (TCS, Southampton, PA). The OEC were determined in 0.1 M potassium phosphate buffer, pH 7.0 at 20°C). The sigmoidally shaped curves demonstrate the normal cooperativity of oxygen binding (Figs. 6A and 6C). The P_{50} value, which measures the partial pressure of oxygen at which hemoglobin is half-saturated, was determined for Hb AS1 and Hb AS2 and compared with Hb A and Hb F (Table 1). The P_{50} for Hb AS1 is slightly elevated, but within the normal range, and this hemoglobin responds normally to the allosteric effector 2,3-diphosphoglycerate (2,3-DPG); that is, oxygen affinity is decreased in the presence of 2 mM 2,3-DPG (Fig. 6B). The P_{50} for Hb AS2 is slightly lower than normal (6.7 mm Hg) but 2,3-DPG raises

this value to 8.4 mm Hg. The oxygen affinity of Hb AS2 is functionally equivalent to Hb F in the presence of 2 mM 2,3-DPG (Fig. 6D) and, therefore, Hb AS2 should adequately bind and deliver oxygen *in vivo*.

Table 1. P_{50} values for recombinant and naturally-occurring human hemoglobins

Sample	P_{50} (mm Hg)	
	without DPG	with DPG
Hb AS1	10.5	15.0
Hb AS2	6.7	8.4
Hb A	8.7	13.3
Hb F	8.8	10.0

Anti-Sickling Properties of AS1 and AS2 hemoglobins

Hb S (100%) or mixtures of Hb S (75%) and Hb A, AS1, AS2, or F (25%) were deoxygenated and polymerization as a function of time was measured spectrophotometrically as the temperature of the hemoglobin solution was raised from 0°C to 30°C (Adachi et al., J. Biol. Chem. 254:7765, 1979; Adachi et al., J. Biol. Chem. 255:7595, 1980; Kinetics of polymerization were determined in 1.8 M potassium phosphate buffer. Polymerization was initiated using the temperature jump method in which the temperature of deoxygenated hemoglobin solutions is rapidly changed from 0°C to 30°C and the time course of aggregation is monitored turbidimetrically at 700 nm). Fig. 7A shows that Hb S polymerizes relatively rapidly and that Hb A, AS1, AS2, and F delay Hb S polymerization to different extents. Hb AS1 inhibits Hb A polymerization more efficiently than Hb A; however, Hb

AS1 inhibits much less effectively than Hb F which is known to inhibit sickling *in vivo* at a 3:1 ratio (Noguchi et al., New Eng. J. Med. 318:96, 1988). Finally, Hb AS2 inhibits Hb S polymerization at approximately the same level as Hb F. This result strongly suggests that Hb AS2 will inhibit Hb S polymerization *in vivo* if expression of AS2 at a level of 25% of total hemoglobin can be achieved.

The delay times determined in Fig. 7A were all measured at a concentration of 60 mg/dl. Fig. 7B shows the results of similar experiments performed at variable concentrations of total hemoglobin. The ratio of Hb S to Hb A, AS1, AS2 or F in all of these experiments was 3:1. In this figure the log of the reciprocal of the delay time and the log of hemoglobin concentration are plotted. As reported previously (Hofrichter et al., Proc. Natl. Acad. Sci. USA 71:4864, 1974; Wishner et al., J. Mol. Biol. 98:179, 1975; Crepeau et al., Nature 274:616, 1978; Dykes et al., J. Mol. Biol. 130:451, 1979; Padlan et al., J. Biol. Chem. 260:8280, 198; and Eaton et al., Blood 70:1245, 1987), an empirical relationship between delay time and hemoglobin concentration can be described by the following equation: $1/td=\gamma S^n$, where $S=[Hb]total/[Hb]soluble$, and γ is an experimental constant. The n value is related to the size of nuclei formed during polymerization. The n values of the data shown in Fig. 7B are between 2 and 3, which agree well with those shown previously in high phosphate buffer (Adachi et al., J. Biol. Chem. 254:7765, 1979). At higher concentrations of hemoglobin, the delay times for Hb AS2 and Hb F overlay indicating that Hb AS2 and Hb F have virtually identical anti-polymerization activity.

The results described above demonstrate that the genetic modification of two surface amino acids in Hb A produces a unique human hemoglobin (Hb AS2) that inhibits

Hb S polymerization as effectively as Hb F. As discussed above, the β-globin LCR enhances β-globin gene expression much more effectively than γ-globin gene expression in adult erythroid cells. Therefore β^{AS2}, which is a β-globin gene with the anti-polymerization properties of γ-globin, is a useful molecule for use in the method of the present invention.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Prchal, Josef T.
Luhovy, Marian
McCune, Steven L.
Prchal, Jaraslov F.

(ii) TITLE OF INVENTION: TRANSDUCTION OF GENES INTO HUMAN HEMATOPOIETIC STEM CELLS USING RECOMBINANT ADENO-ASSOCIATED VIRAL VECTORS

(iii) NUMBER OF SEQUENCES: 3

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

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(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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What is claimed is:

1. An adeno-associated viral vector made in a semi-packaging cell line, and comprising a nucleic acid encoding a therapeutic gene product, for use in therapy.

2. A hematopoietic cell comprising an adeno-associated viral vector made in a semi-packaging cell line, and comprising a nucleic acid encoding a therapeutic gene product, for use in therapy.

3. Use of an adeno-associated viral vector for the preparation of a medicament for use in therapy for a disease of hematopoietic cells in a mammal, said viral vector being made in a semi-packaging cell line and comprising a nucleic acid encoding a gene product capable of alleviating a symptom of said disease.

4. A hematopoietic cell comprising an adeno-associated viral vector for use in therapy for a disease of hematopoietic cells in a mammal, said adeno-associated viral vector being made in a semi-packaging cell line and comprising a nucleic acid encoding a gene product capable of alleviating a symptom of said disease.

5. The use of claim 2 or 4, wherein said hematopoietic cell comprising said adeno-associated viral vector is a hematopoietic stem cell.

6. The use of claim 3 or 4, wherein said disease is a hemoglobinopathy, Fanconis' anemia, adenosine deaminase deficiency, or acquired immune deficiency syndrome.

7. The use of claim 6, wherein said hemoglobinopathy is sickle cell anemia or β-Thalassemia.

8. The use of claim 1, 2, 3, or 4, wherein said gene product is an anti-sickling hemoglobin, β-globin, fetal γ-globin, the gene product of the Fanconis' Anemia C gene, adenosine deaminase, a ribozyme that cleaves HIV RNA, or RNA that is antisense to HIV RNA.

9. The use of claim 1, 2, 3, or 4, wherein said adeno-associated viral vector is produced from said semi-packaging cell line at a titer of at least 10^7 or 10^8 plaque-forming units/milliliter.

10. The use of claim 1, 2, 3, or 4, wherein said semi-packaging cell line comprises the rep or cap genes of adeno-associated virus on an episomal vector.

11. A hematopoietic stem cell comprising an adeno-associated viral vector made in a semi-packaging cell line and comprising a nucleic acid encoding a gene product capable of alleviating a symptom of a disease of hematopoietic cells.

12. The hematopoietic stem cell of claim 11, wherein said gene product is an anti-sickling hemoglobin, β-globin, fetal γ-globin, the gene product of the Fanconis' Anemia C gene, adenosine deaminase, a ribozyme that cleaves HIV RNA, or RNA that is antisense to HIV RNA.

13. The hematopoietic stem cell of claim 11, wherein said adeno-associated viral vector is produced from said semi-packaging cell line at a titer of at least 10^7 or 10^8 plaque-forming units/milliliter.

14. The hematopoietic stem cell of claim 11, wherein said semi-packaging cell line comprises the rep

or cap genes of adeno-associated virus on an episomal vector.

15. An adeno-associated viral vector made in a semi-packaging cell line and comprising a nucleic acid encoding a gene product capable of alleviating a symptom of a disease of hematopoietic cells.

16. The vector of claim 15, wherein said gene product is an antisickling hemoglobin, β-globin, fetal γ-globin, the gene product of the Fanconis' Anemia C gene, adenosine deaminase, a ribozyme that cleaves HIV RNA, or RNA that is antisense to HIV RNA.

17. The vector of claim 15, wherein said adeno-associated viral vector is produced from said semi-packaging cell line at a titer of at least 10^7 or 10^8 plaque-forming units/milliliter.

18. The vector of claim 15, wherein said semi-packaging cell line comprises the rep or cap genes of adeno-associated virus on an episomal vector.

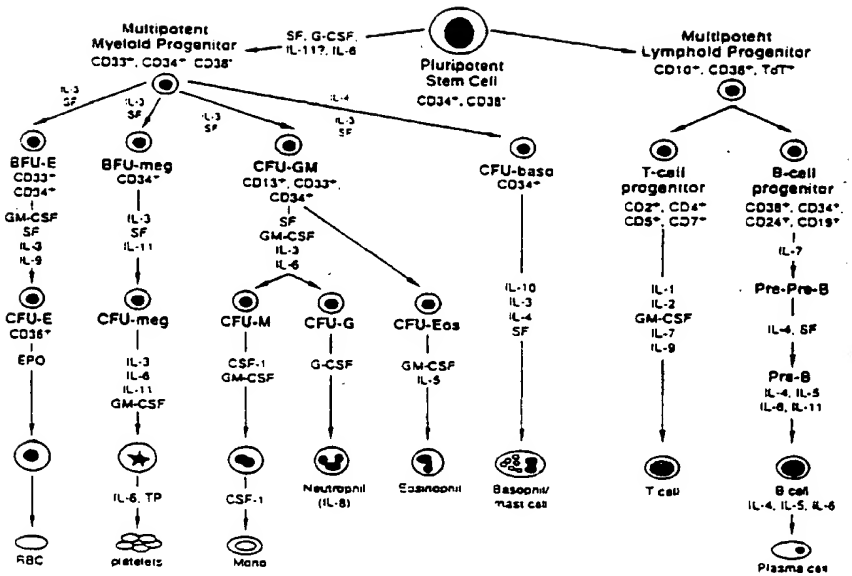


FIG. 1

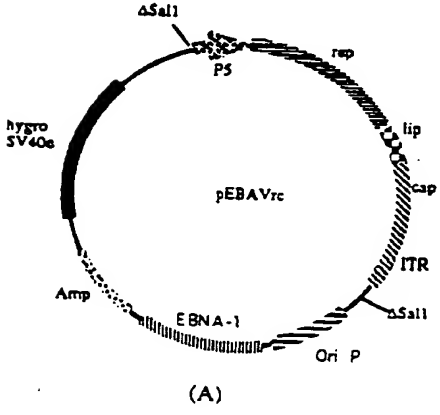


FIG. 2A

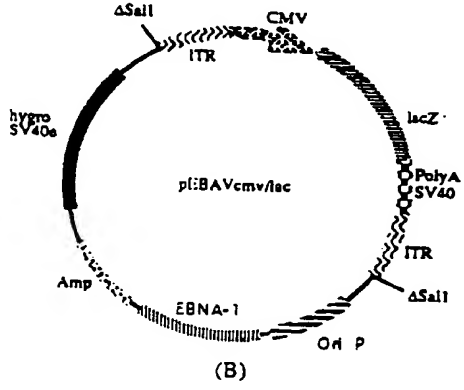


FIG. 2B

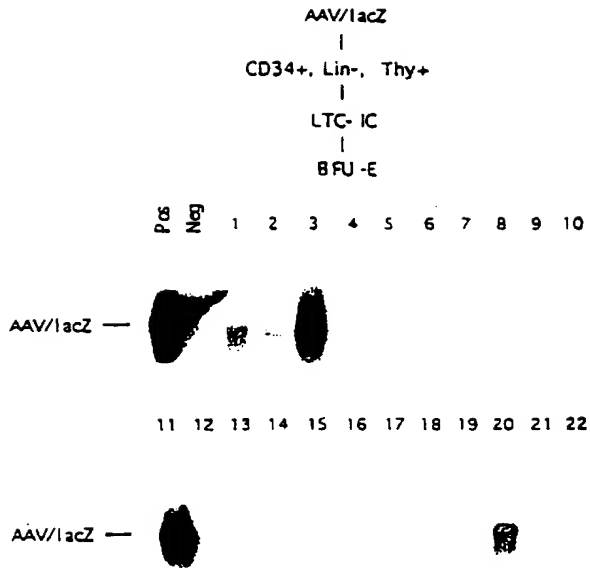

AAV/lacZ
CD34+, Lin-, Thy+
LTC-IC
BFU-E
Pos
Neg
1 2 3 4 5 6 7 8 9 10
AAV/lacZ
11 12 13 14 15 16 17 18 19 20 21 22
AAV/lacZ

FIG. 3

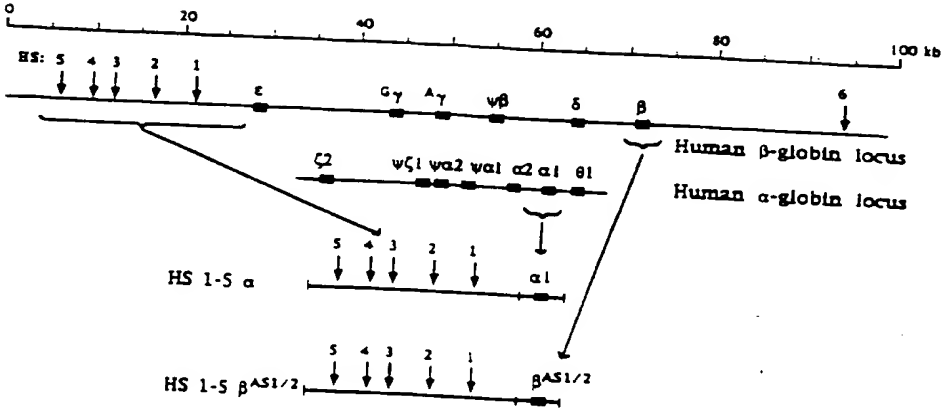

0
20
40
60
80
100 kb
HS: 5 4 3 2 1
ε
Gγ
Aγ
ψβ
δ
β
6
Human β-globin locus
ζ2
ψζ1 ψα2 ψα1 α2 α1 θ1
Human α-globin locus
HS 1-5 α
α1
HS 1-5 βAS1/2
βAS1/2

FIG. 4

FIG. 5A

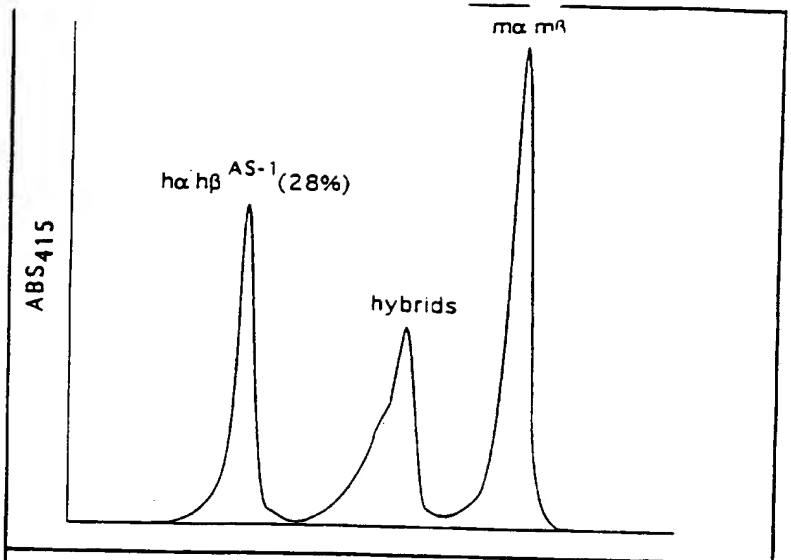


FIG. 5B

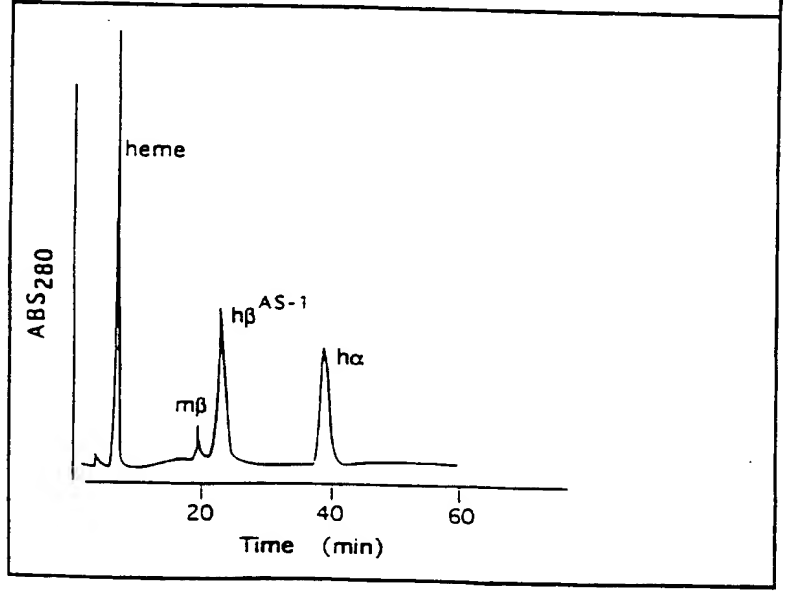


FIG. 5C

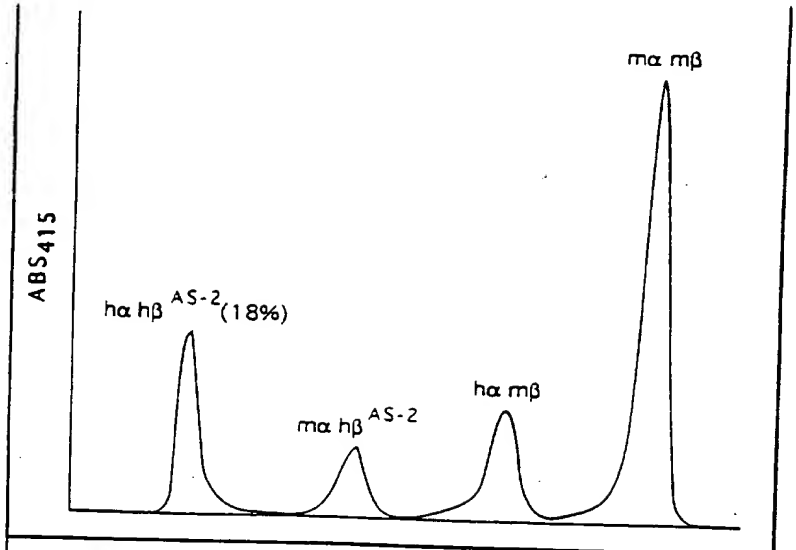

ABS415
hα hβ AS-2 (18%)
mα hβ AS-2
hα mβ
mα mβ

FIG. 5D

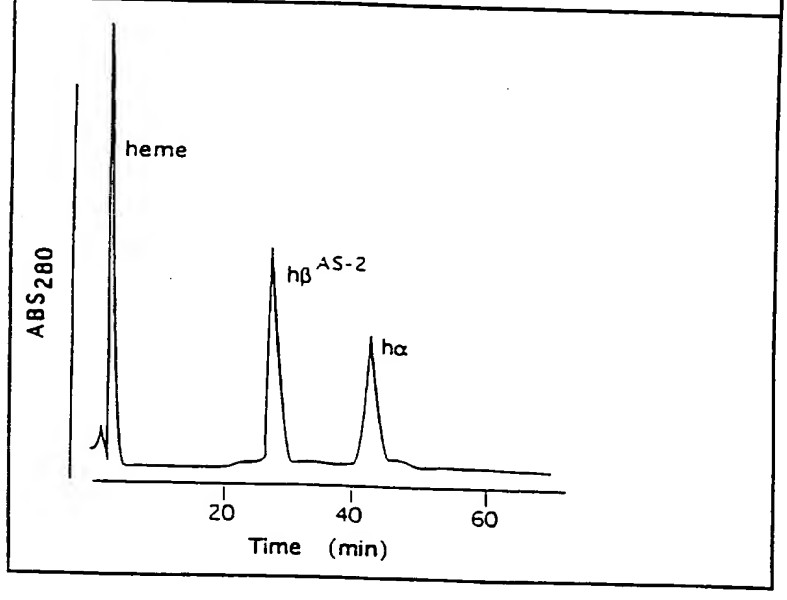

ABS280
heme
hβ AS-2
hα
20
40
60
Time (min)

FIG. 6A

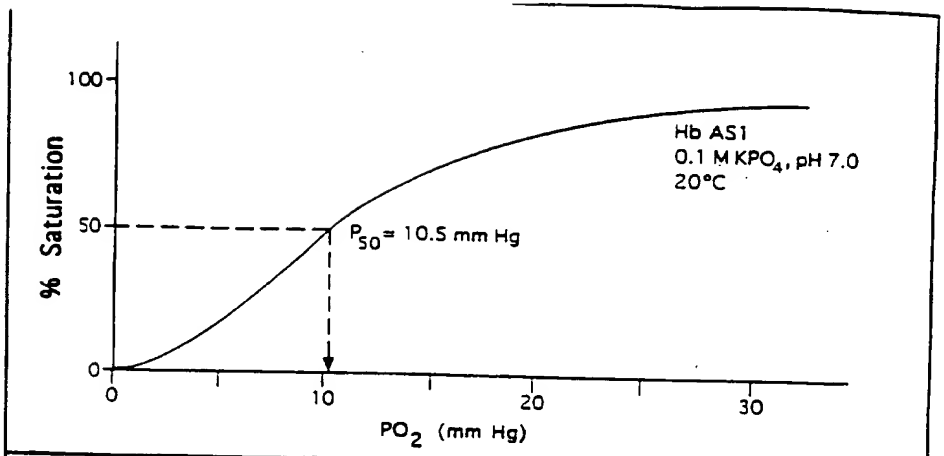


FIG. 6B

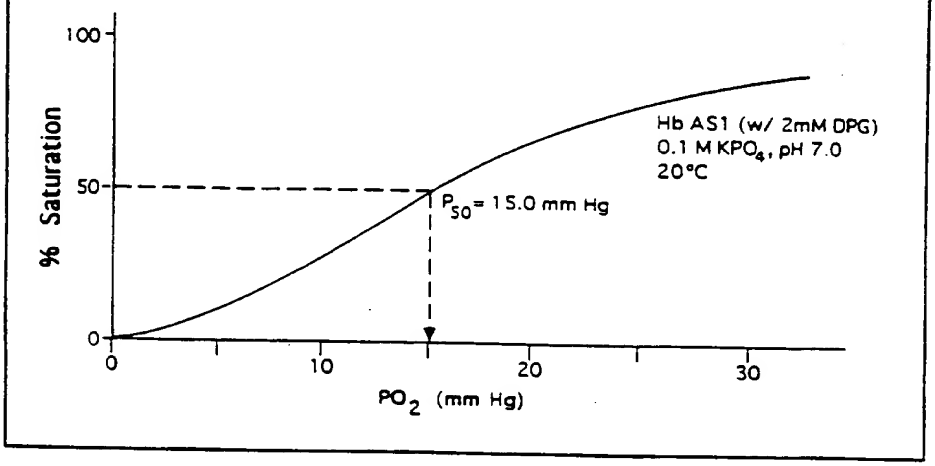


FIG. 6C

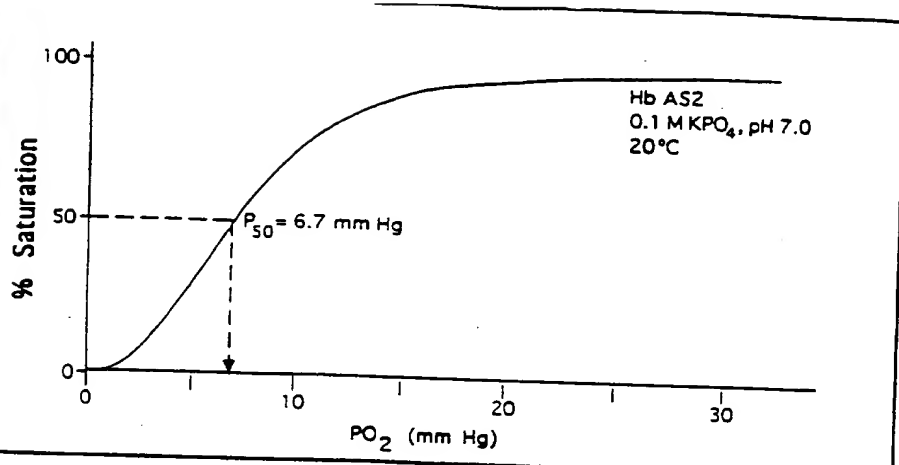
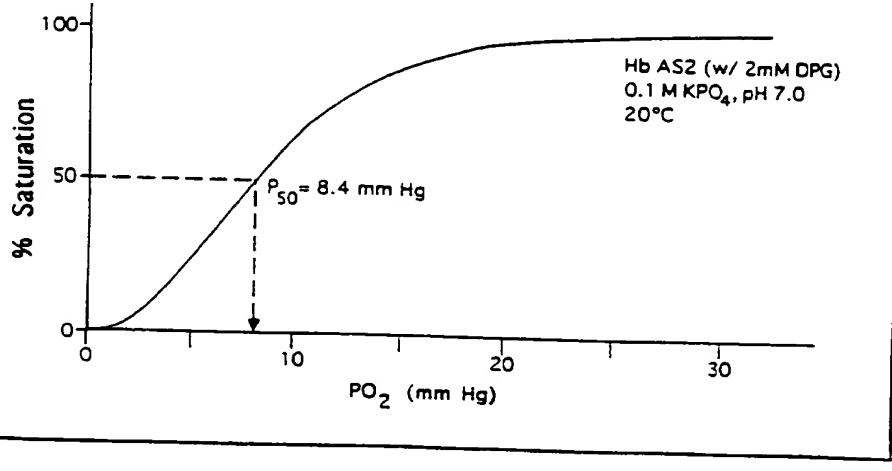


FIG. 6D



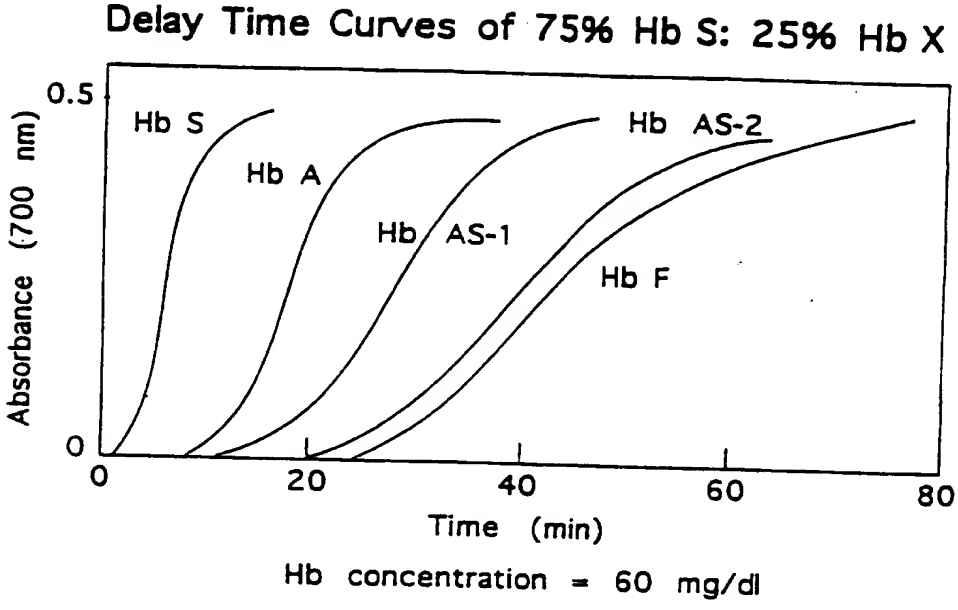
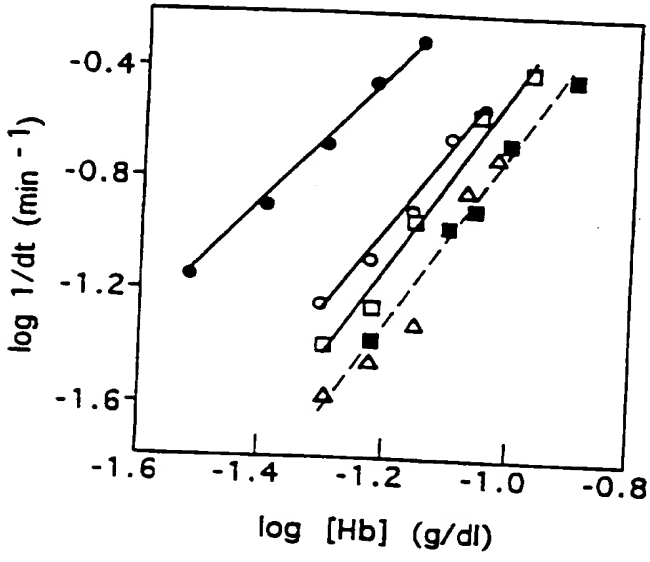


FIG. 7A

Delay Time vs. Hemoglobin Concentration



● 100% Hb S
○ 75% Hb S/25% Hb A
□ 75% Hb S/25% Hb AS1
■ 75% Hb S/25% Hb AS2
△ 75% Hb S/25% Hb F

FIG. 7B

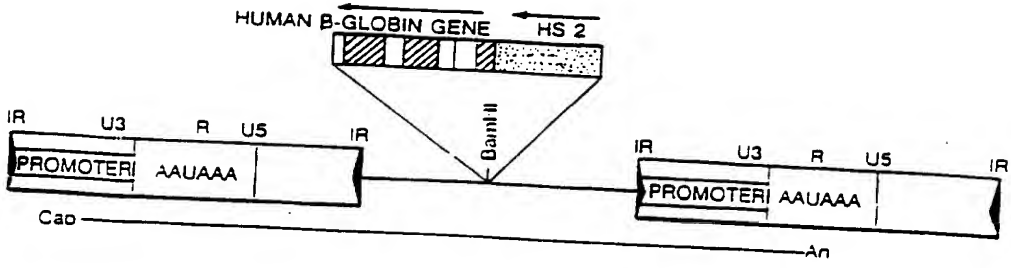


FIG. 8

INTERNATIONAL RCH REPORT

Interna[illegible]l application No.
PCT/US95/11858

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/320.1; 424/93.1+; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 424/93.1+; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, USPAT, EMBASE, BIOSIS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	C ion of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The Lancet, Volume 339, issued 21 March 1992, GUTIERREZ ET AL, "Gene Therapy for Cancer", pages 715-721, see entire document.	1-18
X --- Y	Virology, Volume 162, issued 1988, LAFACE ET AL, "Gene Transfer Into Hematopoietic Progenitor Cells Mediated by an Adeno-Associated Virus Vector", pages 483-486, see entire document, especially pages 483-484.	1 --- 2-18
X --- Y	J. Exp. Med., Volume 179, issued June 1994, ZHOU ET AL, "Adeno-associated Virus 2-mediated High Efficiency Gene Transfer into Immature and Mature Subsets of Hematopoietic Progenitor Cells in Human Umbilical Cord Blood", pages 1867 1875, see entire document, especially pages 1867 and 1873.	1 --- 2-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 NOVEMBER 1995	24 JAN 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Andrew Milne
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11858

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,252,479 (SRIVASTAVA) 12 October 1993, see entire document.	1-18

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL [redacted]RCH REPORT

Internat[redacted] application No.
PCT/US95/11858

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/00, 15/09, 15/63, 15/70, 15/74; A01N 63/00; A61K 51/00, 51/02